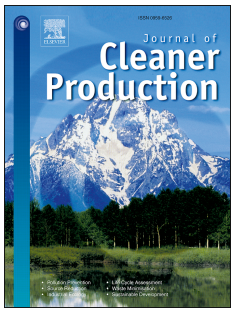

Article:
McElroy, Con Robert orcid.org/0000-0003-2315-8153, Attard, Thomas Michael, Farmer, Thomas James orcid.org/0000-0002-1039-7684 et al. (4 more authors) (2017) Valorization of spruce needle waste via supercritical extraction of waxes and facile isolation of nonacosan-10-ol. Journal of Cleaner Production. 10. pp. 557-566. ISSN 0959-6526

https://doi.org/10.1016/j.jclepro.2017.10.002

Accepted Manuscript



Valorization of spruce needle waste via supercritical extraction of waxes and facile isolation of nonacosan-10-ol

Con R. McElroy, Thomas M. Attard, Thomas J. Farmer, Alicia Gaczynski, David Thornthwaite, James H. Clark, Andrew J. Hunt

PII: S0959-6526(17)32295-3

DOI: 10.1016/j.jclepro.2017.10.002

Reference: JCLP 10794

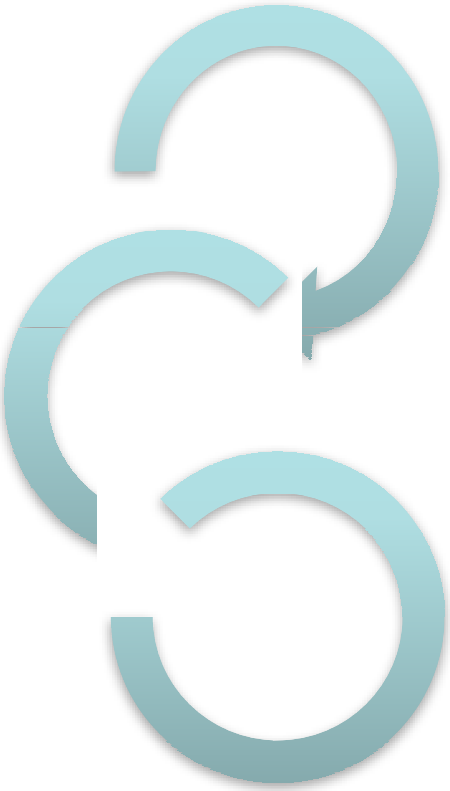
To appear in: *Journal of Cleaner Production*

Received Date: 7 July 2017

Revised Date: 1 September 2017

Accepted Date: 1 October 2017

Please cite this article as: McElroy CR, Attard TM, Farmer TJ, Gaczynski A, Thornthwaite D, Clark JH, Hunt AJ, Valorization of spruce needle waste via supercritical extraction of waxes and facile isolation of nonacosan-10-ol, *Journal of Cleaner Production* (2017), doi: 10.1016/j.jclepro.2017.10.002.



Word count: 6810 words

Valorization of spruce needle waste via supercritical extraction of waxes and facile isolation of nonacosan-10-ol

Con R. McElroy,[a] Thomas M. Attard, [a] Thomas J. Farmer, [a] Alicia Gaczynski, [a] David Thornthwaite,[b] James H. Clark,[a] and Andrew J. Hunt*[a]

[a] Green Chemistry Centre of Excellence, Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK. Email: andrew.hunt@york.ac.uk, Telephone: +44 (0)1904 324456.

[b] Unilever Research and Development Port Sunlight Laboratories, Quarry Road East, Bebington, Wirral, UK CH63 3JW

Keywords: Spruce, Supercritical, Nonacosanol, Wax, Extraction, Separation, Biorefinery

Abstract

Supercritical carbon dioxide was utilized as a sustainable alternative to solvent extraction of waxes from the waste needles of two spruce species, namely Norwegian and Sitka spruce. These extracts were rich in nonacosan-10-ol, an organic compound with hydrophobic properties that lends its use in the preparation of superhydrophobic coatings. The highest crude yields were 1.7% *w/w* of needles obtained at 400 bar and

60 °C , while nonacosan-10-ol was selectively extracted at 200 bar and 60 °C (8070 ±91.1 µg/g of needles). Purification of nonacosan-10-ol from the wax extracts was conducted using a simple rapid green recrystallization technique. This yielded a recovery of 44.6% ±2% and 48.4% ±2% of the total nonacosan-10-ol from the original crude Sitka (3600 µg/g of needles) and Norwegian wax (1920 µg/g of needles) respectively. Application of nonacosan-10-ol to a filter paper led to the formation of highly hydrophobic surfaces, with preliminary contact angles of up to 149^{o}. This sustainable production method may develop opportunities to valorize forestry waste within a holistic biorefinery.

1. Introduction

Wood is a valuable resource that has been utilized for centuries in a wide variety of applications including construction, paper and as a source of chemicals (Arshadi et al., 2012 & 2013; Attard et al., 2016a). Wood based biorefineries currently exist in parts of Scandinavia and North America. However, a greater utilization of waste residues is necessary to make a biorefinery truly holistic (Arshadi et al., 2016; Attard et al., 2015a, 2016a,b; Budarin et al., 2011). Therefore, research must be carried out to systematically tailor and select 'green sustainable' processes to isolate, extract and analyze different chemicals from low-value tree waste fractions with considerable content of extractives (Backlund et al., 2014; Miranda et al., 2012). Needles from forestry wastes are one of the examples that currently constitute a waste resource as they are not utilized by the forestry industry.

Although exploitation of forest residues would lead to a reduction in waste and utilization of renewable resources, there has been very little attention given to valorize this potential feedstock. This has led to significant accumulation of overproduced biomass from neglected forests that have little or no use, which is not only a waste but could also pose as a major fire risk.

The extractives found in the needles have a host of bio-derived chemicals that could potentially be utilized in a number of industrial applications including hydrophobic coatings (Attard et al., 2015a; Backlund et al., 2014). There has been considerable interest in studying the superhydrophobicity of plant surfaces due to their potential wide applications in self-cleaning, drag reduction, anti-sticking, anti-icing and so on (Bhushan and Jung, 2011; Chen et al., 2012). The definition for superhydrophobicity is when a drop on a surface has a contact angle above 150^{o} (Guo and Liu, 2007). The major property of superhydrophobic surfaces is their ability to repel water. An important factor to superhydrophobicity is the chemical composition of the epicuticular waxes covering the aerial tissues of the plant coupled with the micro-/nano-hierarchical structure of the cuticle (Bhushan et al., 2009; Wang et al., 2014). In lotus leaves, the strong water repellency is due to wax tubules composed of the secondary alcohols nonacosan-10-ol and nonacosanediols (Ensikat et al., 2011). Nonacosan-10-ol is present in many natural superhydrophobic surfaces including lotus leaves and conifer needles, and it has significant potential for its use in coatings for porous materials. However, this molecule is currently not commercially exploited. Since nonacosan-10-ol comprises up to 60% of the total wax found in the needles of conifer species (Matas et al., 2003), it could be used as an alternative to the currently utilized non-renewable coatings, such as

plastic coatings on porous materials. Extraction of nonacosan-10-ol from spruce offers several distinct advantages. Firstly, nonacosan-10-ol is the most abundant wax found in spruce needles (Simmleit and Schulten, 1989). Secondly, the growth rate of spruce trees is very fast (Macmillan, 1991), with a yield class (mean cubic meters growth) for Sitka spruce of 14 (i.e. 14 cubic meters per hectare per year according to Forestry Commission (Forestry Commission, 2017)). In terms of maximum timber potential, Sitka spruce requires only 40 – 60 years, whereas oak trees require 150 years (Forestry Commission, 2017). Spruce comprises 29% of all UK commercial forestry, which covers over 1,000,000 hectares (Mason and Perks, 2011; Moore, 2011), resulting in a high turnover and large quantities of needles. Thirdly, as previously stated, spruce needles currently constitute a waste stream and have no commercial value.

The extraction of epicuticular waxes from agricultural wastes (Attard et al., 2015a,b & 2016b), as well as nonacosan-10-ol from Ephedra herbs (Choi et al., 1996), have already been shown to be effective utilizing supercritical carbon dioxide ($scCO_2$) as a renewable solvent . $ScCO_2$ offers numerous advantages over conventional solvent extraction in that the selectivity towards target molecules could be achieved by fine-tuning the solvent power (McHugh and Krukonis, 1994; Özcan and Özcan, 2004). This is carried out simply by changing the temperature and pressure of the solvent (Lang and Wai, 2001; Vilegas et al., 1997; Zougagh et al., 2004). $ScCO_2$ leaves no solvent residues and is regarded as a non-toxic solvent (Hunt et al., 2010). Furthermore, $scCO_2$ has been shown to be effective in improving the downstream processing of biomass in a biorefinery, whereby increased sugar yields have been reported for various biomass types, as well as significantly improved off-gassing from wood pellets (Attard et al.,

2015b, 2016a, b). This recent work indicates that $scCO_2$ can be used effectively for valorizing forestry waste, generating bio-derived chemicals as well as improving downstream processing. Optimization studies on wax extraction from spruce species have not been previously conducted. To date, reported purification of nonacosan-10-ol involved time and material intensive chromatographic techniques, which utilize toxic solvents, in particular $CHCl_3$ and benzene (Jetter and Riederer, 1994; Matas et al., 2003; Yao et al., 2007).

Herein, this work focuses on the supercritical extraction of waste spruce needles that are rich in the secondary alcohol nonacosan-10-ol. The extraction, optimization and characterization of waxes from two species of spruce namely Sitka Spruce and Norwegian Spruce have been carried out for the first time using $scCO_2$ as a green alternative solvent. More importantly, a facile green recrystallization technique was conducted in order to isolate the nonacosan-10-ol from the complex mixture of lipophilic molecules utilizing a highly scalable method. To the authors' best knowledge, combination of supercritical extraction followed by the use of a facile recrystallization technique for the recovery of nonacosan-10-ol has not yet been reported.

2. Materials and Method

2.1 Biomass and sample preparation

With the kind support of the Forestry Commission, Sitka Spruce was collected from Dalby Forest at North Yorkshire in the United Kingdom, while the Norwegian Spruce was collected from Umeå, Sweden. The biomass constituted the needle-rich small branches from numerous trees that had been recently felled for lumber. Samples of the

biomass were then separated, through air drying until a constant weight was observed (*circa* three weeks) and a small portion refrigerated at 5 ^{o}C. A small sample of the dry biomass and all of the refrigerated wet biomass were then milled as a whole, while the needles of the remaining dry feedstock were easily separated by shaking from the branch. All milling was carried out using a Glen-Creston mill, with a 2 mm mesh.

2.2 ScCO_2 extraction of spruce needle wax

A Thar SCF500 CO_2 extractor was used to carry out the extractions. The dried, milled biomass (50 g) was placed into the extraction cylinder and extracted for 2 hours with CO_2 at various pressures (200, 300 and 400 bar) and temperatures (40, 50 and 60 ^{o}C), with a flow rate of 40 g min^{-1}. The extract was depressurized to atmospheric conditions into the first extraction vessel and the wax removed using dichloromethane (2 x 50 mL washes). The solvent was evaporated to yield the product.

2.3 Purification of nonacosan-10-ol from scCO_2 extracted spruce needles (150 g)

Methanol (50 ml) was added to the spruce needle extract (3.22 g). The solution was stirred at 50 ^{o}C for 10 minutes and left to cool to room temperature. The solution was filtered and washed with cold methanol (2 x 10 ml) to obtain the crude product. The crude product was then dissolved in hot methanol (30 ml) yielding a light green solution and a dark green wax/dense oil. The solute (light green solution) was decanted and left to cool yielding a light green precipitate. The dark green wax/dense oil was washed with hot methanol (10 ml) to yield a brittle wax. The light green precipitate was recrystallized a second time in hot methanol (30 ml), decanted, left to cool, filtered,

144 washed with cold methanol (2 x 10 ml) and dried to yield a white precipitate,
145 nonacosan-10-ol. Unrecovered nonacosan-10-ol could be recovered from the
146 recrystallization media by evaporation of the methanol solvent, allowing this crude
147 mixture to potentially be recycled within the recrystallization process.
148
149 [Scheme 1 here]
150
151 2.4 Contact angle method
152 Basic contact angle measurements were obtained by dissolving a known amount of pure
153 nonacosan-10-ol in hot methanol to give a 1% or 20% by weight solids content. A solid
154 support (glass or filter paper) was then dipped in the solution, removed and left to dry
155 under atmospheric conditions. Once dry, a drop of distilled water was applied to the
156 surface using a pasture pipette with a minimum drop height without the pipette coming
157 into contact with the surface material. Photos of the droplet were then taken and the
158 contact angle was determined.
159
160 2.5 Derivitization prior to High Temperature-Gas Chromatography (HT-GC) analysis
161 Derivitization was achieved by the addition of 200 µl N,O-*bis*-(trimethylsilyl)-trifluoro-
162 acetamide with 1% trimethylchlorosilane to 30 mg of the crude extract dissolved in 1 ml
163 toluene. The solution was placed in an oven and heated at 75 ^{o}C for 45 minutes.
164
165 2.6 HT-GC procedure for analysis of wax
166 High temperature Gas Chromatography was conducted using an Agilent Technologies
167 6890N Network GC System. This was fitted with a ZB-5HT capillary column

(dimensions: 30 m x 250 µm x 0.25 µm nominal) at constant pressure (22.35 psi). A temperature of 300 ^{o}C was selected as the injector temperature and flame ionization detector temperature while the carrier gas utilized was helium. A split ratio of 5:1 was applied. Injection of the sample (1 µl injection volume) was carried out by automated injection. The oven temperature was set as follows: (i) Initial temperature of 60 ^{o}C, held for 1 minute ii) The temperature was increased to 360 ^{o}C at a ramp rate of 8 ^{o}C min^{-1} iii) The temperature was held at 360 ^{o}C for 30 minutes.

2.7 HT-GC-MS (High Temperature-Gas chromatography Mass Spectrometry) procedure for wax analysis

A Perkin Elmer Clarus 500 GC coupled with a CLarus 500 quadrupole mass spectrometer was used to perform the high temperature-gas chromatography mass spectrometry. A DB5HT capillary column was fitted (dimensions: 30 m x 250 µm x 0.25 µm nominal) at constant pressure (22.35 psi). A temperature of 300 ^{o}C was selected as the injector temperature and helium was selected as the carrier gas. The flow rate was 1.2 ml min^{-1}. The temperature profile for the oven was as follows: (i) Initial temperature of 60 ^{o}C, held for 1 minute ii) The temperature was increased to 360 ^{o}C at a ramp rate of 8 ^{o}C min^{-1} iii) The temperature was held at 360 ^{o}C for 30 minutes. The electron ionization mode (EI) at 70 eV was selected for the Clarus 500 quadrupole mass spectrometer with a source temperature of 300 ^{o}C. A scan range of 30 – 1200 amu per second was applied.

3. Results and Discussion

3.1 Optimization of the supercritical extraction of waxes from Spruce needles

191 An attempt was made to optimize the % yield of wax extracted from the spruce needles
192 using $scCO_2$ extraction by applying the factorial experimental design, whereby
193 temperature and pressure (independent variables) were varied in order to study the
194 effect this has on the extraction yield (dependent variable). The experiments required 2^f
195 runs (f = factors), where each factor was at two levels, those of the minimum and
196 maximum extraction limits.
197
198 A variety of temperatures and pressures were utilized in an experimental 2x2 plot
199 (supplementary Figure S1) in order to investigate two parameters at the same time. A
200 pressure range of 200 to 400 bar was applied (since previous studies have shown that
201 very low pressures give low yields of extract) while a temperature range of 40 to 60 ^oC
202 was applied. Four experimental points were selected at maximum and minimum
203 temperatures and pressures (A, B, C and D – Figure S1). A center point was also
204 introduced in order to ensure there was no risk of missing a non-linear relationship
205 within the experimental range.
206
207 The impact of pressure and temperature was modelled by means of a dimensionless
208 factor coordinate system, whereby "-1" was assigned for the low level and "+1" was
209 given to the high level for each parameter. The center point was assigned a coordinate
210 value of "0" (coincides with the origin of the system) as shown in Tables 1 and 2 below.
211
212 [Table 1 here]
213 [Table 2 here]
214

Therefore, five experiments were conducted for the optimization study and multiple linear regression (MLR) was used in order to deduce the relationship between crude yield and temperature and pressure. The first order polynomial function utilized for the MLR is shown below in Equation 1:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$$

Equation 1: First order polynomial function

Where Y is the % crude yield, b_1 and b_2 correspond to the major effects of the coordinates x_1 (temperature) and x_2 (pressure), b_0 represents the center point yield (E – the response at "0" level) and b_{12} is the second order interaction term. Two-hour extraction times were allotted for each set of experiments and a flow rate of 40 g min^{-1} was applied. Table 3 summarizes the % yield of wax obtained at different temperatures and pressures.

[Table 3 here]

[Figure 1 here]

From the % yields shown in Table 3, MLR was conducted as shown in Equations 2-5 below in order to obtain a first order polynomial function to model the $scCO_2$ extraction of waxes from Sitka spruce needles.

$$b_0 = \frac{1}{4}(y_1 + y_2 + y_3 + y_4)$$

$$b_1 = \frac{1}{4}(-y_1 + y_2 - y_3 + y_4)$$

$$b_2 = \frac{1}{4}(-y_1 - y_2 + y_3 + y_4)$$

$$b_{12} = \frac{1}{4}(y_1 - y_2 - y_3 + y_4)$$

Equations 2, 3, 4 and 5: coefficient calculations for the first order polynomial function

$$Y = 1.32 + 0.24x_1 + 0.16x_2 - 0.015x_1x_2$$

Equation 6 First order polynomial function for the $scCO_2$ extraction of waxes from needles.

The coefficients of pressure, temperature and the second order interaction term are shown in equation 6, and can be used to help understand the effect of temperature and pressure (as well as the combined effect of the two parameters) on the extraction process. The theoretical % yield for the center point value E (1.32%) was found to be in good correlation with the experimental value (1.41%) (a 0.09% difference with a 6.8% error) indicating the model behaves well for this extraction. It can be seen that in this instance the value of x_1 for temperature is higher than that of x_2 (pressure) which indicates that temperature has a higher influence on the extraction yield than pressure and density (since an increase in pressure at constant temperature leads to an increase in density).

Figure 1 demonstrates a 2-D plot highlighting the variation in % crude yield of wax with varying temperature and pressure (the different % crude yields may also be viewed in Figure S2). As shown in Table 1, the % yield of wax extracted from the Spruce needles under the different conditions applied varied from 0.91 to 1.70%.

The dielectric constant and density of CO_2 are dictated by temperature and pressure (Hunt et al., 2010). In the extraction of wax the density of CO_2 is an important factor. Higher yields were obtained at 400 bar 40 °C than at 200 bar 40 °C, indicating that the increase in density led to a greater yield, this is consistent with other wax extraction

studies (Attard et al., 2015b; Sin et al., 2014). However, the highest yields (1.70%) were achieved using a pressure of 400 bar and temperature of 60 °C, where the density is lower than that at 400 bar and 40 °C. This demonstrates that even though density has an important role, there are other factors such as temperature that dictate the solubility of compounds in CO_2. These results are consistent with the findings of the first order polynomial function. Studies have highlighted that higher yields can be obtained when the temperature is close to or above the waxes melting point (Sin et al., 2014). Since wax is in semi-crystalline form, higher temperatures enable the melting of the wax and therefore aiding in extraction. Furthermore, an increase in temperature at constant pressure results in vapor pressure increase leading to an increase in solute solubility in $scCO_2$.

The results show that a significant increase in yield was observed at elevated temperatures, where the extraction yields at 200 bar and 40 °C were 0.91% rose to 1.41% when increased by 20 °C. Furthermore, at high pressure conditions a significant increase in yield was obtained at higher temperatures (i.e. 1.70% at 400 bar and 60 °C –), as compared to lower temperatures (i.e. 1.26%, at 400 bar and 40 °C –). This data correlates to the first order polynomial function obtained, whereby temperature is the most influential factor on the % yield (though pressure also has a positive influence).

3.2 Characterization and quantification of lipophilic compounds in the needle extracts from Sitka Spruce.

GC and GC-MS analyses were used to characterize the underivatized and silylated extracts using a high temperature capillary column and methods which allowed for the elution and determination of high-molecular weight compounds such as sterols and unsaturated long-chain ketones.

Results from Table 4 and Figure 2 showed that the major compounds identified were found to be nonacosan-10-ol, free saturated (ranging from C_{12} to C_{20} in chain length) and unsaturated fatty acids (C_{18} chain length), unsaturated ketones (C_{28} and C_{30} chain length), sterols, hydroxyacids, benzoic acid and phytol. For all conditions examined, it was found that nonacosan-10-ol was the predominant compound in the wax extracts. Although conditions of 400 bar and 60 °C gave the highest % crude extract yield, it was found that the conditions which led to the highest yields of noncosan-10-ol were 200 bar and 60 °C, with approximately 8,070 ± 91.1 μg/g needles extracted. This is also consistent with the observation that a high % crude yield of wax was extracted using these conditions. The lowest quantities of nonacosan-10-ol were extracted when using conditions of 200 bar and 40 °C, with approximately 2,870 ±266.6 μg/g of needles extracted. When using the conditions of 200 bar and 60 °C, the highest yields of β-sitosterol and benzoic acid were also obtained, with an estimated 398 ± 6.6 and 100 ± 16.6 μg/g of needles extracted respectively. Conditions of 300 bar and 50 °C led to the highest yields of ketones, with approximately 978 ± 81.3 μg/g of needles extracted. The same conditions led to the highest extraction of fatty acids and hydroxyacids. Therefore, it can be concluded that, although conditions of 400 bar and 60 °C led to the highest % crude yield of wax extract, the largest quantities of noncosan-10-ol were achieved with 200 bar and 60 °C. Thus, the conditions needed for the extraction vary according to the desired product, i.e. the extract as a whole or nonacosan-10-ol or unsaturated ketones.

306

307 **[Table 4 here]**

308 **[Figure 2 here]**

309

310 Furthermore, nonacosan-10-ol is the major compound, constituting around 60% of the
311 total extract at 200 bar and 60 °C. For all other extracts, nonacosan-10-ol constitutes
312 considerably low proportion of the composition (i.e. 22 – 42%).

313

314 3.3 Characterization and quantification of lipophilic compounds in the needle extracts
315 from Norwegian Spruce.

316

317 Since conditions of 200 bar and 60 °C led to the highest quantities of nonacosan-10-ol
318 from Sitka Spruce, these conditions were also applied to the extraction of wax from
319 Norwegian spruce needles in order to make a direct comparison of the nonacosan-10-ol
320 content between the two species. When compared to the Sitka, Norwegian spruce
321 exhibited a more complex mixture of lipophilic chemicals (as seen in Figure S3). There
322 is a wider variety of fatty acids, steroids and also a number of terpenoid compounds,
323 which are absent or below the level of detection in the Sitka spruce.

324

325

326 Figure 3 compares the major compounds found in the waxes extracted from the Sitka
327 spruce and Norwegian spruce. Nonacosan-10-ol concentrations in Sitka spruce needles
328 are approximately double the amount present in the Norwegian spruce needles, 8070
329 ±91.1 µg/g of needles and 3966.6 ±114.3 µg/g of needles respectively. On the other

hand, significantly larger amounts of saturated and unsaturated fatty acids are present in the Norwegian spruce needles (2122.4 ±20 µg/g of needles and 3669.3 ±19.1 µg/g of needles respectively) compared to the Sitka spruce needles (551.8 ±37 µg/g of needles and 181.42 ±20.3 µg/g of needles respectively). Sitosterol is the only steroidal compound found in the Sitka spruce, while three other steroidal compounds are found in the Norwegian spruce (9,19-cyclolanostan-3-ol, 24 methylene - (3β-)-, 24-Methylenecycloartan-3-one and Stigmastan-3,5-diene) which accounts for the greater concentration of these compounds in the Norwegian spruce extracts (2122.4 ±43.6 µg/g of needles). Unsaturated ketones are present in the needle extracts of both species; however, a higher abundance is found in the Sitka spruce (885.1 ±20.1 µg/g of needles) when compared to the Norwegian spruce (159.6 ±0.9 µg/g of needles) (Table 5).

[Table 5 here]

[Figure 3 here]

3.4 Simple isolation and purification of Nonacosan-10-ol from spruce

The development of new separation technologies for biorefineries is of significant importance for their long-term development and commercial success. Due to the complex and highly functionalized nature of bio-derived molecules, traditional techniques such as distillation are not always suitable for retaining functionality. Therefore, new or greener methods that preserve the complexity of the bio-derived molecules are of vital importance. Furthermore, standard chromatographic separation techniques such as HPLC and continuous liquid chromatography are energy intensive

and use large quantities of solvents leading to cumulative solvent waste which is often problematic to dispose(Yao et al., 2007). Therefore, a simple and efficient isolation and purification methodology for noncosan-10-ol was developed. The placing of the crude product obtained by extraction in a polar solvent causes most lipophilic compounds to crash out. The initial polar solvents screened were methanol, ethanol and iso-propanol. These polar solvents were selected as they are labelled as 'Recommended' on the recent Chem 21 solvent selection guide and Sanofi selection guide; whereas these had only some issues on the GSK solvent selection guide (associated with health, flammability and explosion) (Henderson et al., 2011; Prat et al., 2013&2015).

The purest product was obtained using methanol as solvent for purification, where the ratio of methanol to crude extract used was much smaller (12:1), resulting in the formation of a green precipitate (Figure S14). Methanol has the advantages of being relatively inexpensive, potentially bioderived, easily biodegradable and has low resistivity (Prat et al., 2013). However, the drawbacks of methanol are flammable and volatile (Prat et al., 2013). After filtration, this green precipitate could, in turn, be solvated in hot methanol to produce a light green solution and dark green black wax. The hot solute was then carefully decanted into separate glassware and left to cool, where a light green precipitate formed upon cooling. This was recrystallized a second time to yield a white precipitate. GC-MS analysis (shown in Figure S4 and S5) of the white precipitate confirmed it to be nonacosan-10-ol, however minor impurities are still present. The purity of the nonacosan-10-ol obtained was found to be 90% (Figure S6). Proton and carbon NMR of the product matched literature values, although other signals are also present, again indicating the presence of minor impurities (as shown in Figures

S7 and S8). Evaporation of methanol could also be utilized to recover additional nonacosan-10-ol.

In addition to the nonacosan-10-ol, a brittle dark green wax was also obtained. GC-FID analysis of this brittle wax reveals that the sample contains nonacosan-10-ol, two trace fatty acids and predominantly two compounds. As shown in Figures S9 and S10, the GC-MS EI fragmentation patterns suggest these two compounds to be C_{28} and C_{30} unsaturated aldehydes, giving molecular ions of 406 and 434 respectively, with no other fragments observed, relating to compounds with molecular formulas of $C_{28}H_{54}O$ and $C_{30}H_{58}O$. Figure S11 shows the proton NMR of the compound, with evidence of unsaturation visible in the spectrum. However, the distinctive signal of the aldehyde proton is missing, which shows that these compounds are more likely to be unsaturated ketones. Additional unrecovered nonacosan-10-ol in this sample could be recovered through recycling of this crude mixture within the recrystallization process.

In order to ensure repeatability as well as broad application, the same purification technique was conducted on the more complex Norwegian spruce wax extract (Figure 4). Once again three fractions were obtained, each differing in composition. The methanol-soluble layer was found to be rich in terpenes, fatty acids, phenolic compounds and sterols. These molecules are completely absent or found in minute quantities in the other fractions showing the selective extraction of these molecules in methanol. A dark green/black wax was also obtained with the Norwegian spruce extract, which consists mainly of unsaturated ketones, saturated aldehydes and wax esters. Importantly, the same result was obtained with the Norwegian spruce extract as

with the Sitka spruce extract, i.e. a white precipitate was collected following the purification method which was confirmed to be nonacosan-10-ol by GC-FID. This indicates that, although Norwegian spruce had a more diverse and complex range of lipophilic molecules, the purification method still led to the selective isolation of nonacosan-10-ol of reasonably high purity. This shows that the purification method is not limited to just one type of biomass extract but can be applied to different wax extracts containing high amounts of nonacosan-10-ol.

Mass balances were calculated for each wax extract and it was found that approximately 44.6% and 48.4% ± 2% of the total nonacosan-10-ol were recovered from the original crude Sitka and Norwegian wax respectively. As shown in Figure 4, some of the nonacosan-10-ol was lost during the first step due to its limited solubility in methanol (as shown in Figure 4.) while some of it was also found present in the ketone layer. Nevertheless, substantial amounts of nonacosan-10-ol were isolated using this simple technique, equating to approximately 3,600 µg/g needles for the Sitka spruce and 1,920 µg/g needles for the Norwegian spruce. Recycling of the methanol and recycling the dark green brittle wax to undertake additional recrystallizations could yield yet more nonacosan-10-ol (Figure S14).

[Figure 4 here]

Therefore, it has been shown that a simple single solvent purification technique could be used to obtain nonacosan-10-ol of relatively high purity. This would reduce

considerably the volumes of solvent used and the time of separation when compared to standard chromatographic techniques. Furthermore, since only one solvent is used, it can be recycled without risk of contamination.

3.5 Scanning Electron Microscopy (SEM)

The surface of the original biomass and supercritically extracted biomass, along with nonacosan-10-ol and wax residue were investigated by scanning electron microscope (Figure 5). Figure 5A and 5B show the nanotubules formed by nonacosan-10-ol and observed on the needles. Figure 5C and 5D shows that these self-assembled nano structures have partially survived the milling process and are present in the biomass feedstock prior to extraction. . Figure 5E and 5F demonstrate the purified nonacosan-10-ol compound and despite the rapid recrystallisation process complex spherical structures, which indicates self-assembly phenomenon was observed. Additionally SEM images of the biomass post extraction showed no remaining wax indicating all surface wax had been successfully removed (figure S12).

[Figure 5 here]

3.6 Simple application of the nonacosan-10-ol extract

Initial testing of nonacosan-10-ol to demonstrate its use in potential barrier property applications was achieved by its coating onto a porous material. To this end, a 1% nonacosan-10-ol solution in methanol was applied to a glass slide. Nonacosan-10-ol solution in methanol (1% and 20%) were applied to porous materials, namely filter paper. As shown in Figure 6, droplets formed on the glass and filter paper. The contact angles were measured and are reported in Table 6.

[Figure 6 here]

[Table 6 here]

A droplet of water was then applied to the coated surface and the contact angle was measured (Figure 6). In the case of the glass (Figure 6C), i.e. the presence of a 1% nonacosan-10-ol increases the contact angle from 37^{o} (control – untreated slide) to 128^{o} for the coated slide (Figure 6D), indicating markedly increased water barrier properties. For the filter paper, it can be observed that a 1% nonacosan-10-ol solution increased the contact angle from 0^{o} to 132^{o} while a 20% nonacosan-10-ol solution resulted in a contact angle of 149^{o}, indicating a hydrophobic surface which borders on being superhydrophobic. Optical Microscopy imaging and SEM show the nonacosanol assembling on glass slide (Figure S13 and Figure 6F respectively). These preliminary tests demonstrate significant promise and future work will optimize the process to obtain superhydrophobic coatings for utilization in various applications.

4. Conclusions

Therefore, it has been shown that a natural hydrophobic molecule, with potential industrial applications in coatings, could be selectively extracted from forestry waste using clean technology ($scCO_2$) and purified using a simple single solvent technique resulting in significant reductions in solvent usage, considerably lower volumes of solvent waste and hence a more efficient process.

Extraction of Spruce using $scCO_2$ extraction yielded 1.70% of wax at 400 bar and 60 °C, nonacosan-10-ol was the major component at 200 bar and 60 ° C. Purification of nonacosan-10-ol from the wax was conducted using a simple, green recrystallization technique with a purity of 90%. Preliminary results on contact angle measurements show coating of paper with 20% nonacosan-10-ol solution resulted in a highly hydrophobic surface with contact angle of 149^{o}. This method may develop new opportunities to selectively extract and purify nonacosan-10-ol using green technologies and solvents from a forestry waste to generate additional value as part of a holistic biorefinery. Finally, valorization of the forest waste would reduce the problem of significant accumulation of overproduced biomass residues from neglected forests.

Acknowledgements

Authors from the University of York would like to acknowledge the financial support of Unilever and the Formas CETEX project. Also, the authors would like to thank Dr Vitaliy Budarin for his assistance in the creation of composite images within this manuscript and Dr Meg Stark for her assistance with SEM analysis.

References

Arshadi, M., Attard, T.M., Bogel-Lukasik, R.M., Brncic, M., da Costa Lopes, A.M., Finell, M., Geladi, P., Gerschenson, L.N., Gogus, F., Herrero, M., Hunt, A.J., Ibanez, E., Kamm, B., Mateos-Aparicio Cediel, I., Matias, A., Mavroudis, N., Montoneri, E., Morais, A.R.C., Nilsson, C., Papaioannou, E.H., Richel, A., Ruperez, P., Skrbic, B.,

497 Bodroza-Solarov, M., Svarc-Gajic, J., Waldron, K., Yuste, F., 2016. Pre-treatment and
498 extraction techniques for recovery of added value compounds from wastes throughout
499 the agri-food chain. Green Chemistry 18, 6160-6204.
500 Arshadi, M., Backlund, I., Geladi, P., Bergsten, U., 2013. Comparison of fatty and resin
501 acid composition in boreal lodgepole pine and Scots pine for biorefinery applications.
502 Industrial Crops and Products 49, 535-541.
503 Arshadi, M., Hunt, A.J., Clark, J.H., 2012. Supercritical fluid extraction (SFE) as an
504 effective tool in reducing auto-oxidation of dried pine sawdust for power generation.
505 RSC Advances 2, 1806-1809.
506 Attard, T.M., Arshadi, M., Nilsson, C., Budarin, V.L., Valencia-Reyes, E., Clark, J.H.,
507 Hunt, A.J., 2016a. Impact of supercritical extraction on solid fuel wood pellet properties
508 and off-gassing during storage. Green Chemistry 18, 2682-2690.
509 Attard, T.M., McElroy, C.R., Gammons, R.J., Slattery, J.M., Supanchaiyamat, N.,
510 Kamei, C.L.A., Dolstra, O., Trindade, L.M., Bruce, N.C., McQueen-Mason, S.J.,
511 Shimizu, S., Hunt, A.J., 2016b. Supercritical CO2 Extraction as an Effective
512 Pretreatment Step for Wax Extraction in a Miscanthus Biorefinery. ACS Sustainable
513 Chemistry & Engineering 4, 5979-5988.
514 Attard, T.M., McElroy, C.R., Rezende, C.A., Polikarpov, I., Clark, J.H., Hunt, A.J.,
515 2015a. Sugarcane waste as a valuable source of lipophilic molecules. Industrial Crops
516 and Products 76, 95-103.
517 Attard, T.M., Theeuwes, E., Gomez, L.D., Johansson, E., Dimitriou, I., Wright, P.C.,
518 Clark, J.H., McQueen-Mason, S.J., Hunt, A.J., 2015b. Supercritical extraction as an
519 effective first-step in a maize stover biorefinery. RSC Advances 5, 43831-43838.

520 Backlund, I., Arshadi, M., Hunt, A.J., McElroy, C.R., Attard, T.M., Bergsten, U., 2014.
521 Extractive profiles of different lodgepole pine (Pinus contorta) fractions grown under a
522 direct seeding-based silvicultural regime. Industrial Crops and Products 58, 220-229.
523 Bhushan, B., Jung, Y.C., 2011. Natural and biomimetic artificial surfaces for
524 superhydrophobicity, self-cleaning, low adhesion, and drag reduction. Progress in
525 Materials Science 56, 1-108.
526 Bhushan, B., Jung, Y.C., Koch, K., 2009. Micro-, nano- and hierarchical structures for
527 superhydrophobicity, self-cleaning and low adhesion. Philosophical Transactions of the
528 Royal Society A: Mathematical, Physical and Engineering Sciences 367, 1631-1672.
529 Budarin, V.L., Shuttleworth, P.S., Dodson, J.R., Hunt, A.J., Lanigan, B., Marriott, R.,
530 Milkowski, K.J., Wilson, A.J., Breeden, S.W., Fan, J., Sin, E.H.K., Clark, J.H., 2011.
531 Use of green chemical technologies in an integrated biorefinery. Energy &
532 Environmental Science 4, 471-479.
533 Chen, Y., Zhang, Y., Shi, L., Li, J., Xin, Y., Yang, T., Guo, Z., 2012. Transparent
534 superhydrophobic/superhydrophilic coatings for self-cleaning and anti-fogging. Applied
535 Physics Letters 101, 033701-1 - 033701-4.
536 Choi, Y.H., Kim, J., Noh, M.J., Park, E.M., Yoo, K.-P., 1996. Extraction of epicuticular
537 wax and nonacosan-10-OL fromEphedra herb utilizing supercritical carbon dioxide.
538 Korean J. Chem. Eng. 13, 216-219.
539 Ensikat, H.J., Ditsche-Kuru, P., Neinhuis, C., Barthlott, W., 2011. Superhydrophobicity
540 in perfection: the outstanding properties of the lotus leaf. Beilstein Journal of
541 Nanotechnology 2, 152-161.
542 Forestry Commission England, 2017. Sitka Spruce- picea sitchensis. Edinburgh, UK.
543 https://www.forestry.gov.uk/forestry/infd-5nlej6.

544 Guo, Z., Liu, W., 2007. Biomimic from the superhydrophobic plant leaves in nature:
545 Binary structure and unitary structure. Plant Science 172, 1103-1112.
546 Henderson, R.K., Jimenez-Gonzalez, C., Constable, D.J.C., Alston, S.R., Inglis, G.G.A.,
547 Fisher, G., Sherwood, J., Binks, S.P., Curzons, A.D., 2011. Expanding GSK's solvent
548 selection guide - embedding sustainability into solvent selection starting at medicinal
549 chemistry. Green Chemistry 13, 854-862.
550 Hunt, A.J., Sin, E.H.K., Marriott, R., Clark, J.H., 2010. Generation, Capture, and
551 Utilization of Industrial Carbon Dioxide. ChemSusChem 3, 306-322.Jetter, R., Riederer
552 M., 1994. Epicuticular crystals of nonacosan-10-ol: In-vitro reconstitution and factors
553 influencing crystal habits. Planta 195, 257-270.
554 Lang, Q., Wai, C.M., 2001. Supercritical fluid extraction in herbal and natural product
555 studies — a practical review. Talanta 53, 771-782.
556 Macmillan, D.C., 1991. Predicting the General Yield Class of Sitka Spruce on Better
557 Quality Land in Scotland. Forestry: An International Journal of Forest Research 64,
558 359-372.
559 Mason, B., Perks, M.P., 2011. Sitka spruce (Picea sitchensis) forests in Atlantic Europe:
560 changes in forest management and possible consequences for carbon sequestration.
561 Scandinavian Journal of Forest Research 26, 72-81.
562 Matas, A.J., Sanz, M. J., Heredia, A., 2003. Studies on the structure of the plant wax
563 nonacosan-10-ol, the main component of epicuticular wax conifers. International
564 Journal of Biological Macromolecules 33, 31-35.
565 McHugh, M.A., Krukonis, V.J., 1994. Supercritical fluid extraction: principles and
566 practice. Butterworth-Heinemann, Stoneham, MA, USA, pp.178.

567 Miranda, I., Gominho, J., Mirra, I., Pereira, H., 2012. Chemical characterization of
568 barks from Picea abies and Pinus sylvestris after fractioning into different particle sizes.
569 Industrial Crops and Products 36, 395-400.
570 Moore, J., 2011. Wood properties and uses of Sitka spruce in Britain. Research Report -
571 Forestry Commission. Edinburgh, UK.
572 https://www.forestry.gov.uk/pdf/FCRP015.pdf/%24file/FCRP015.pdf
573 Özcan, A., Özcan, A.S., 2004. Comparison of supercritical fluid and Soxhlet extractions
574 for the quantification of hydrocarbons from Euphorbia macroclada. Talanta 64, 491-
575 495.
576 Prat, D., Pardigon, O., Flemming, H.-W., Letestu, S., Ducandas, V., Isnard, P.,
577 Guntrum, E., Senac, T., Ruisseau, S., Cruciani, P., Hosek, P., 2013. Sanofi's Solvent
578 Selection Guide: A Step Toward More Sustainable Processes. Organic Process Research
579 & Development 17, 1517-1525.
580 Prat, D., Wells, A., Hayler, J., Sneddon, H., McElroy, C.R., Abou-Shehada, S., Dunn,
581 P.J., 2015. CHEM21 selection guide of classical- and less classical-solvents. Green
582 Chemistry 18, 288-296.
583 Simmleit, N., Schulten, H.-R., 1989. Thermal degradation products of spruce needles.
584 Chemosphere 18, 1855-1869.
585 Sin, E.H.K., Marriott, R., Hunt, A.J., Clark, J.H., 2014. Identification, quantification
586 and Chrastil modelling of wheat straw wax extraction using supercritical carbon
587 dioxide. Comptes Rendus Chimie 17, 293-300.
588 Vilegas, J.H.Y., de Marchi, E., Lancas, F.M., 1997. Extraction of low-polarity
589 compounds (with emphasis on coumarin and kaurenoic acid) from Mikania glomerata
590 ('guaco') leaves. Phytochemical Analysis 8, 266-270.

591 Wang, G., Guo, Z., Liu, W., 2014. Interfacial Effects of Superhydrophobic Plant
592 Surfaces: A Review. Journal of Bionic Engineering 11, 325-345.
593 Yao, S., Liu, R., Huang, X., Komng, L., 2007. Preparative isolation and purification of
594 chemical constituents from the root of *Adenophora tetraphlla* by high-speed counter-
595 current chromatography with evaporative light scattering detection. Journal of
596 Chromatography A 1139, 254-262.
597 Zougagh, M., Valcárcel, M., Ríos, A., 2004. Supercritical fluid extraction: a critical
598 review of its analytical usefulness. TrAC Trends in Analytical Chemistry 23, 399-405.
599

List of Figures

Scheme 1. Flow diagram illustrating the purification of Nonacosan-10-ol from Spruce.

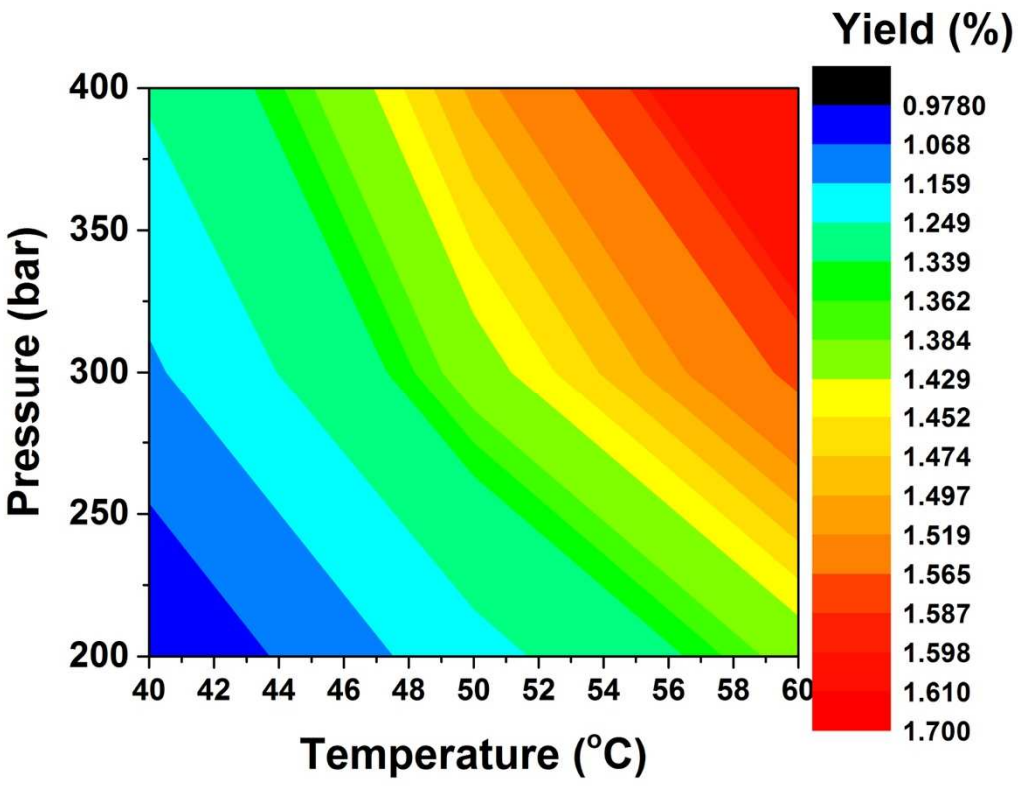


Figure 1. 2-D plot showing the effect of varying pressure and temperature on the % crude wax yield from Sitka Spruce.

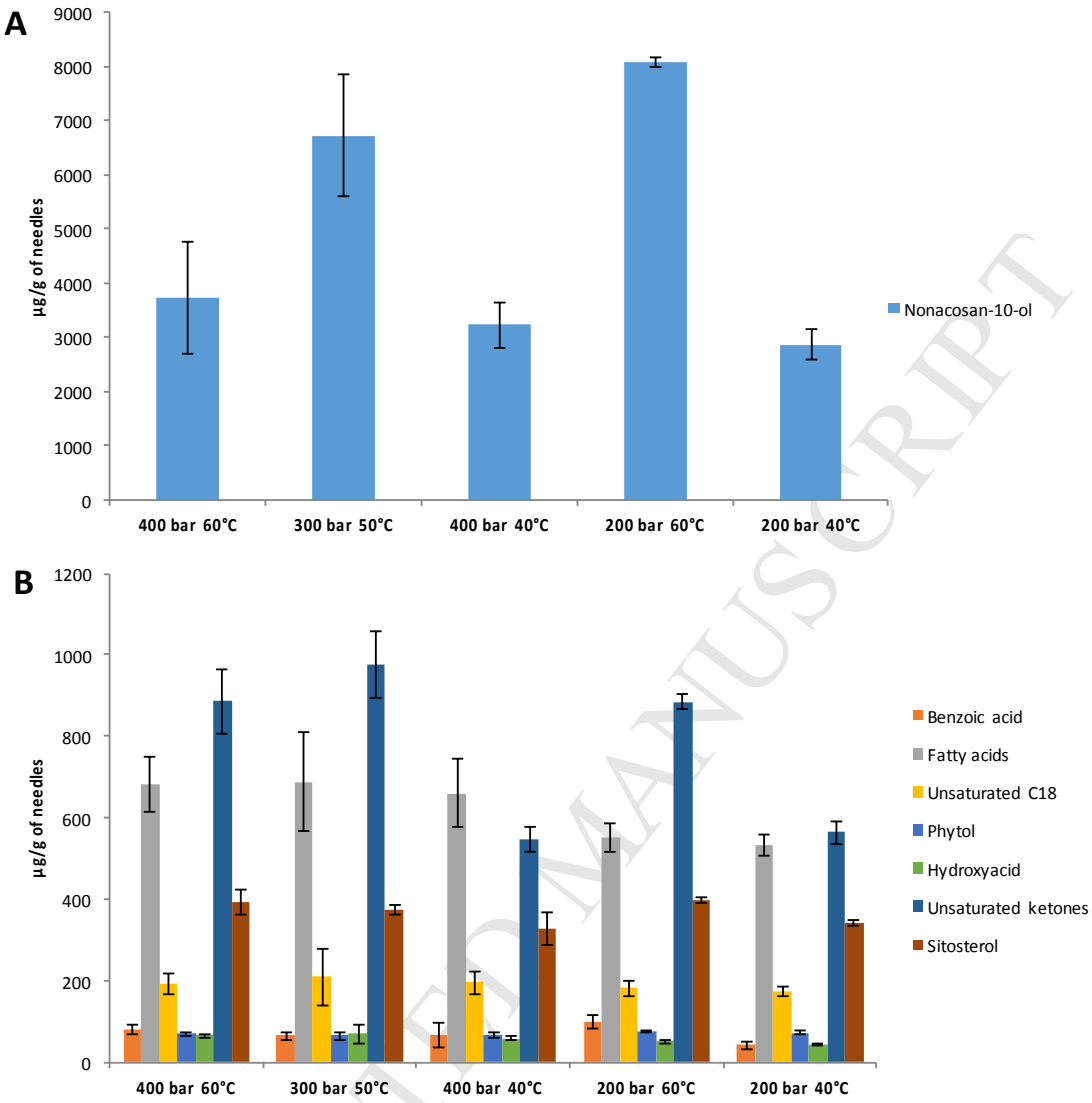


Figure 2. Composition of organic compounds at various temperature and pressure: A) Nonacosan-10-ol B) Benzoic acid, Fatty acids, Unsaturated C18 acids, Phytol, Hydroxyacid, Unsaturated ketones and Sitosterol.

.

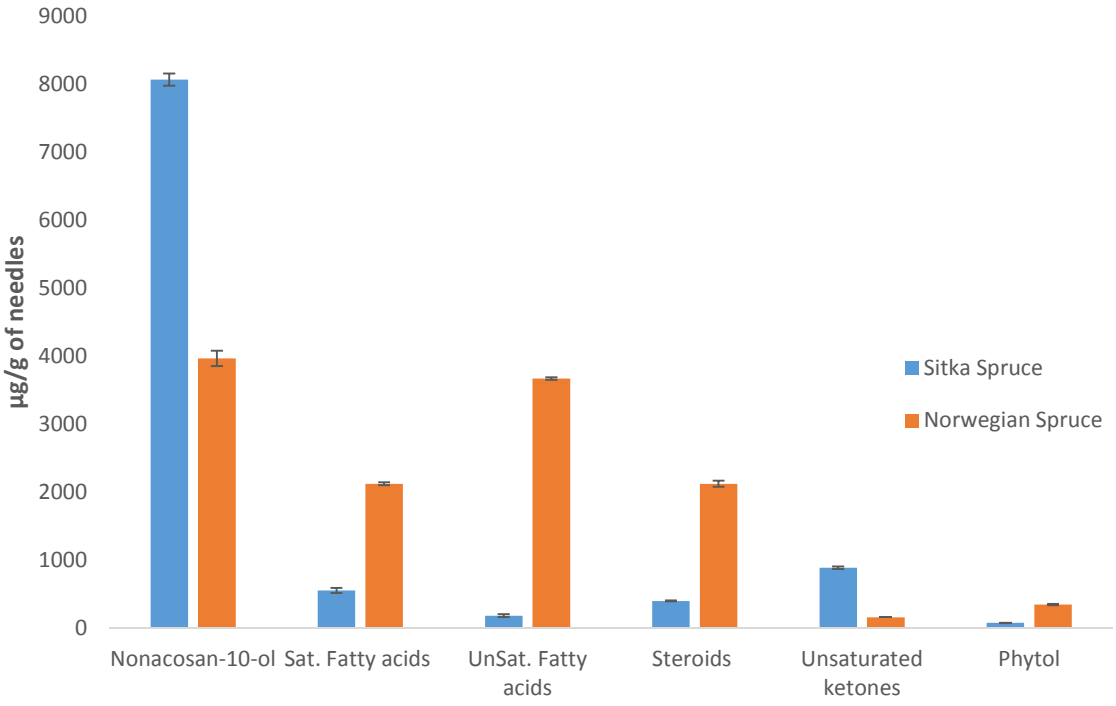


Figure 3. Comparison of major compounds found in waxes from Sitka spruce and Norwegian spruce.

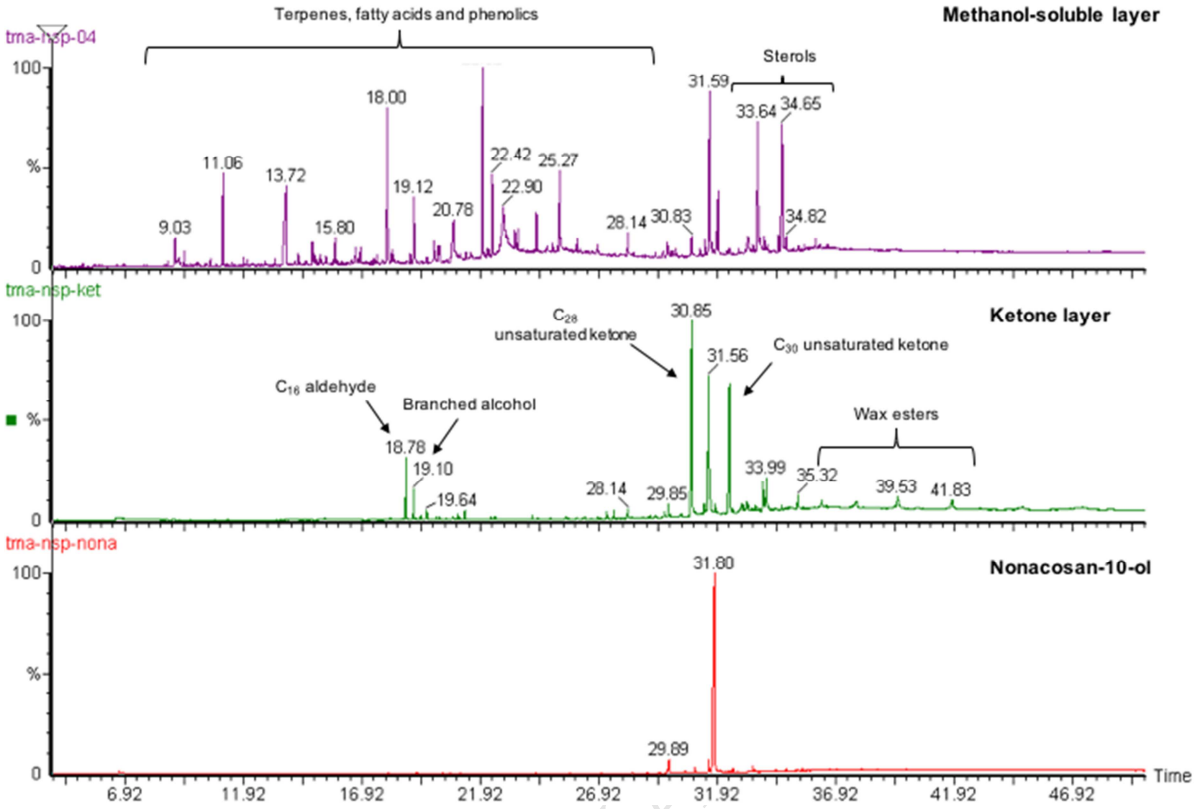


Figure 4. GC-MS chromatograms of a) Methanol-soluble layer b) Ketone layer and c) Nonacosan-10-ol.

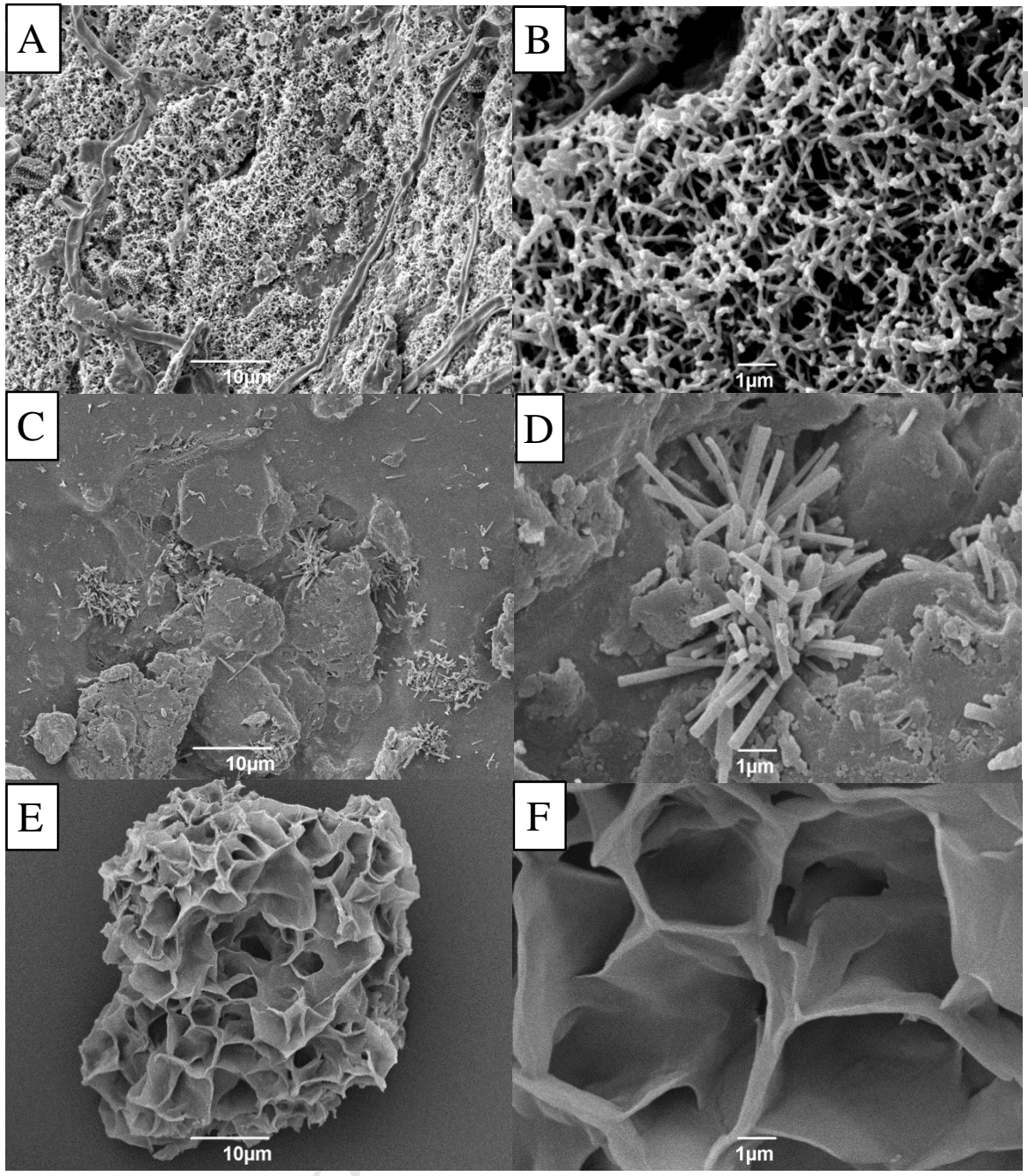


Figure 5. SEM images of spruce and spruce extracts. A and B = Nanotubules formed by nonacosan-10-ol on the biomass (spruce needles), C and D = Nanotubules still present on needles following milling, , E and F = complex, spherical structures of purified nonacosan-10-ol.

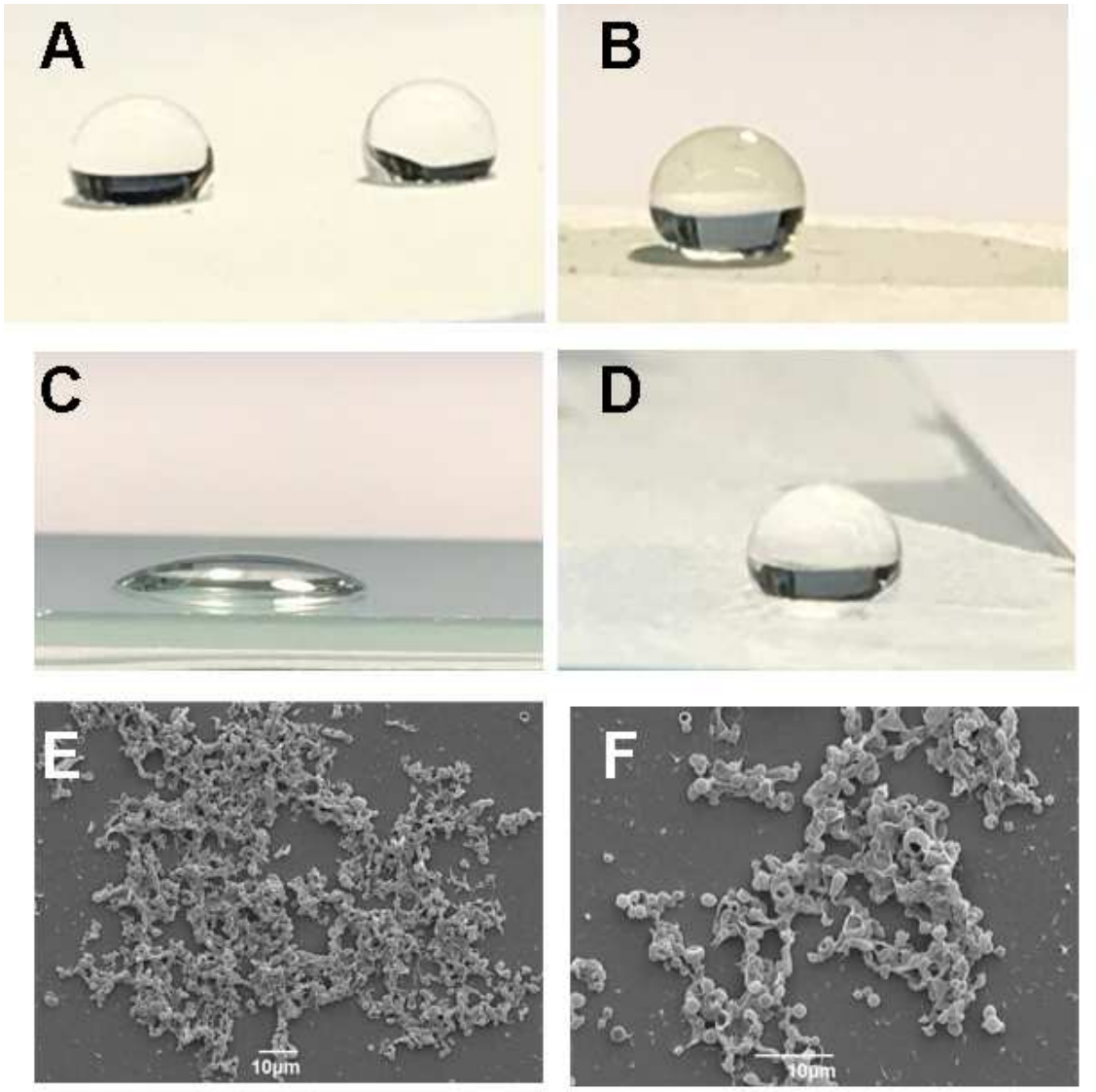


Figure 6 A) Droplets formed on filter paper with a 1% nonacosan-10-ol solution B) Droplet formed on paper with a 20% nonacosan-10-ol solution C) Control: droplet on a glass slide and D) Droplet on glass covered with a 1% nonacosan-10-ol solution. E) SEM of nonacosanol assembling on glass slide (×500) F) SEM of nonacosanol assembling on glass slide (×1,000).

List of Tables

Table 1 The experimental design with the normalized values for temperature and pressure.

Factor	Variable	Normalized values		
		-1	0	1
X1	Temperature (^{o}C)	40	50	60
X2	Pressure (bar)	200	300	400

Table 2. Experimental design with the different conditions and the assigned normalized values.

Experiment Point	Coordinate values		Experimental conditions	
	X1	X2	Temp. (^{o}C)	Pressure (bar)
A	-1	+1	40	400
B	+1	+1	60	400
C	-1	-1	40	200
D	+1	-1	60	200
E	0	0	50	300

Table 3. Extraction yields obtained at different temperatures and pressures for Sitka spruce needles.

Experiment	Temperature (^{o}C)	Pressure (Bar)	Extraction Yield (%)
1	40	200	0.91
2	60	200	1.45
3	60	200	1.36
4	40	400	1.26
5	50	300	1.41
6	60	400	1.70

Table 4. Quantification data of the most abundant compounds found in the wax extracts from spruce under various conditions.

Compounds	$scCO_2$ extraction conditions (°C/bar)				
	40/200 (µg/g of needles)	**60/200 (µg/g of needles)**	**50/300 (µg/g of needles)**	**40/400 (µg/g of needles)**	**60/400 (µg/g of needles)**
Fatty acid					
C12:0	89.6 ±4.2	95.9 ±4.3	109.8 ±25.4	116 ±12.5	104.2 ±5
C14:0	157 ±5.6	157.3 ±8.2	198 ±41.5	210.9 ±24.6	203.8 ±9.9
C16:0	244.6 ±8.8	259.1 ±18.2	294.9 ±74.8	290.1 ±32	284.6 ±25.2
C18 unsat. fatty acids	175.1±11.7	181.4 ±20.3	210.3 ±68.6	196.3 ±27.7	195 ±25.4
C18:0	29.1 ±4.2	27.7 ±4.6	33.2 ±15.2	32.1 ±10.5	33.8 ±9.3
C20:0	13.8 ±1.9	11.9 ±1.7	53.7 ±36.4	11.7 ±3.9	79.9 ±20
Total Fatty acids	709.3 ±36.4	733.2 ±57.3	899.7 ±261.9	857.1 ±111.2	877.5 ±94.8
Fatty alcohols					
Nonacosan-10-ol	2869.8 ±249.1	8070 ±91.1	6718.6 ±1117	3225.3 ±415.8	3719.8 ±1039.2
Unsaturated ketones					
C_{28} + C_{30} Unsat. ketones	563.7 ±27	885.1 ±20.1	978.4 ±81.3	548 ±31.4	885.7 ±79.4
Sterols					
Beta-sitosterol	341.6 ±7.8	397.9 ±6.6	374.5 ±13	329.1 ±39.7	393 ±28.6
Other compounds					
Benzoic acid	42.5 ±8.3	100.2 ±16.6	65.1 ±8.5	67.1 ±28.7	80.9 ±10.3
Hydroxyacid	43.8 ±2.6	51.3 ±5.3	69.5 ±23.6	59.5 ±4.7	65.1 ±8.5
Phytol	74 ±3.1	75.3 ±2.7	65 ±7.2	67.5 ±8.4	70.3 ±3.2

Table 5 Quantification data of the most abundant compounds found in the wax extracts from Norwegian spruce under various conditions.

Compounds	µg/g of needles
Fatty acid	
C12:0	19.3 ±2.6
C14:0	222.4 ±0.8
C16:0	1377.9 ±7.8
C18 unsaturated fatty acids	3669.3 ±19.1
C18:0	156.3 ±7.9
C20:0	122.6 ±0.4
C22:0	223.8 ±0.4
Total Fatty acids	5791.3 ±39.1
Fatty alcohols	
Nonacosan-10-ol	3966.6 ±114.3
Unsaturated ketones	
C28 & C30 Unsaturated ketones	159.6 ±0.8
Sterols	
Beta-sitosterol	1111 ±21.1
9,19-cyclolanostan-3-ol, 24 methylene - (3β-)-	703.5 ±9.4
24-Methylenecycloartan-3-one	59.6 ±1.1
Stigmastan-3,5-diene	248.3 ±12
Total steroid comounds	2122.4 ±43.6
Other compounds	
Borneol	97.7 ±3.5
Bornyl acetate	220 ±27.6
4-hydroxyacetophenone	419.8 ±6.3
Phytol	343.7 ±9.5
Total other compounds	

Table 6. Contact angle measurements for the nonacosan-10-ol coatings on different materials.

Type	**Paper CA**	**Glass CA**
Control	0^{o}	37^{o}
1% Nonacosan-10-ol solution	132^{o}	128^{o}
20% Nonacosan-10-ol solution	149^{o}	-

- Supercritical extraction was employed to valorise waste spruce needles
- Nonacosan-10-ol accounted for 8070 ±91.1 μg/g of needles
- A facile and green recrystallization process isolated 90% pure nonacosanol
- Nonacosanol demonstrated promise as a coating for porous materials
- Highly hydrophobic nonacosanol surfaces exhibited contact angles of 149^{o}